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Comparatively little attention has been paid to the fractional precipitation of antitoxin. Brodie[1] in 1897 separated antitoxin horse serum into four fractions by the progressive addition of ammonium sulphate to half saturation; all four contained, however, relatively equal amounts of antitoxin. Atkinson[2] in this laboratory saturated with sodium chloride a solution of the moist serum globulin precipitate obtained with magnesium sulphate, and by then employing heat differentiated the globulin into several fractions containing antitoxin. The protective properties corresponded roughly to the quantities of serum globulin in the precipitates. In some unpublished experiments he found that alterations of the amounts of coagulated proteid in the several fractions resulted if more magnesium sulphate was added before heating; there were proportionate changes in the distribution of the antitoxin. Owing to the destruction of a portion of the antitoxin at the higher temperature and possible injury by exposing it to heat of less degree, this fractionation must be considered as incomplete and does not exclude a purification of the antitoxin by salt fractionation. The work of E. P. Pick on the ammonium sulphate fractioning of the anti-bodies has been referred to in the preceding communication. Our own experiments have resulted somewhat differently from either those reported by Atkinson or by Pick, and have developed some new and suggestive facts.

On the basis of the solubility of the antitoxic proteids in saturated sodium chloride solution, one of us (Gibson) recently devised a method for the partial purification and concentration

[1] Brodie: *Journ. of Path. and Bact.*, iv, p. 460, 1897.
[2] Atkinson: *Journ. of Exper. Med.*, v, p. 67, 1901.

of antitoxin.[1] This consisted in precipitating the diluted plasma with an equal volume of saturated ammonium sulphate and separating the antitoxic proteids by extracting the precipitate with saturated sodium chloride solution. We now have employed the method of salt fractionation to study further the concentration of antitoxin.

There exists at the present time considerable confusion in comprehending the methods and basic principles of ammonium sulphate fractional precipitation of proteids. The nomenclature which we have employed and which designates the number of cc. of saturated ammonium sulphate solution in 10 cc. of the *precipitated* mixture has been used by some authorities; it avoids the confusion developed by the use of such terms as "per cent $(NH_4)_2SO_4$ solution" and "per cent saturation $(NH_4)_2SO_4$" "per cent of saturated $(NH_4)_2SO_4$ solution" and "per cent saturation $(NH_4)_2SO_4$ solution" and it seems the simplest and best *practical* expression of degrees of saturation yet suggested. We advise that this method be employed in future papers on fractional precipitation.

Mann, in his version of Cohnheim's "*Chemie der Eiweisskörper*," states (p. 292): "As the solubility of ammonium sulphate is 76.8° (per cent ?) at room temperature, it is easy to calculate what percentage of ammonium sulphate is required for bringing about incipient and complete precipitation of any one albumin, as soon as we know what amounts of saturated ammonium sulphate have to be added for any given quantity of fluid." The simplicity of the above method of calculating vanishes when attention is drawn to the fact that while 100 parts of water dissolve 76.8 gms. of dry ammonium sulphate, the volume resulting is increased to 141 cc. so that 100 cc. of the saturated solution actually contain approximately 54 gms. of the salt, and the degree of saturation as indicated by the *content* of dry ammonium sulphate must be calculated with reference to the latter figure. An example will make clearer the above statement: To obtain a concentration of "half saturation" ammonium sulphate, equal volumes of the proteid solution and of saturated ammonium sulphate solution are mixed; according to the apparent meaning of Mann's obscure statement, 100 cc. of the resulting " half saturated" solution would contain 38.4 gms. of the dry salt; it actually does contain 27 gms. of ammonium sulphate.[2]

[1] The literature on the purification and chemical characters of antibodies has been briefly reviewed in a paper on "The Practical Concentration of Diphtheria Antitoxin for Therapeutic Use," this *Journal*, i, p. 161, 1906, and more recently by Ledingham: *Journ. of Hyg.*, vii, p. 65, 1907.

[2] Because of the change in the volume of the solvent on adding the salt, it is similarly not possible to add 38.4 gms. of ammonium sulphate to 100 cc. of water and have a solution at "half saturation." In this case the volume would be increased to 120.7 cc. and 100 cc. would contain 31.7 gms. of the salt.

E. P. Pick has fallen into the same error in his paper on the fractionation of the anti-substances in the globulins of serum. He speaks (p. 356) of the limits of the various serum fractions as follows: "dass das von Reye aus normalen Pferdeserum abgeschiedene Fibrinoglobulin entsprechend einer Sättigung von 21.5 Proz. Ammonsulfat.... ...ein bestimmter Teil (the euglobulin) des nun übrig bleibenden Globulins keine antitoxische Wirkung hatte und dass sich dieser aus dem Serum noch bequem abschieden liess, wenn die Flussigkeit einer Gehalt von 25.6 Proz. an Ammonsulfat enthielt. Es verblieb nunmehr ein Eiweisskörper in Lösung (the pseudoglobulin) der durch weiteres Eintragen der gesättigten Ammonsulfatlösung bis zu einem Gehalte von 38 Proz. von dem Serumalbumin gut zu trennen ist und den Heilkörper in quantitativer Ausbeute enthält." The precipitation limits are distinctly designated here by 21.5, 26.6 and 38 per cents of ammonium sulphate in the precipitated mixture. They actually mean a content of 2.9, 3.33, and 4.9 cc. of saturated ammonium sulphate solution in 10 cc. of the precipitated mixtures, which would then contain, respectively, 15.67, 18.00 and 26.50 gms. of the dry ammonium sulphate per 100 cc.—figures which by no means or method of interpretation can be logically expressed by 21.5, 25.6 and 38 percentages of ammonium sulphate. Fortunately the fault lies in the nomenclature only, the precipitations being accomplished by the use of saturated ammonium sulphate solution.

Twenty liters of plasma (475 units per cc.) were diluted with 20 liters of water; by fractioning with saturated ammonium sulphate solution, the three proteid precipitates were obtained which separated at concentrations corresponding to 3.3 cc., 3.3–3.8 cc. and 3.8–5.0 cc. of the saturated salt solution in 10 cc. The saturated sodium chloride soluble (antitoxic) globulins of these fractions and of the 5.0 saturation precipitate of a second 20 liters of the plasma were prepared as usual. Proteid determinations (coagulations) and potency tests were duplicated.

Prep. 77.

Fractions.	A. 0.0–5.0	B. 0.0–3.3	C. 3.3–3.8	D. 3.8–5.0
Volume cc.	5200	1440	1400	2050
Units per cc.	1450	1150	1350	1750
Times concentrated	3.05	2.42	2.84	3.68
Per cent recovered	79.3	17.4	19.9	37.8
Proteid, gms. per 100 cc.	11.66	11.51	9.87	9.70
Units, per gm. proteid	12436	10000	13666	18000

A second experiment with a 450 unit plasma gave the following results:

Prep. 82.

Fractions.	A. 0.0–5.0	B. 0.0–3.3	C. 3.3–3.8	D. 3.8–5.0
Volume cc.	6240	1350	1640	2550
Units per cc.	1050	900	1300	1600
Times concentrated.	2.34	2.00	2.89	3.56
Per cent recovered.	72.8	13.9	22.6	45.3
Proteid, gms. per 100 cc.	10.59	12.06	13.46	13.41
Units, per gm. proteid.	9914	7464	9655	11930

These observations show that the antitoxic globulins of the higher fraction are much more potent than those of the less soluble proteids.

Both the preparations by the half-saturation ammonium sulphate method and by fractioning, when precipitated from the saturated sodium chloride solution and dialyzed, contained a probably partially denaturalized antitoxic globulin; this had a diminished solubility and antitoxic potency (per gram proteid) and was precipitated on slight acidification by diluting twenty times. The filtrates from the water-acid precipitable globulin coagulated at 73°, while saline solutions of the precipitates so obtained showed varying and much lower coagulating temperatures. The solutions of the high proteid fractions have a peculiar green color. A redetermination of the precipitation limits of the globulin in the three fractions after removal of the water-acid precipitable proteid, showed that the different precipitation limits were relatively characteristic for the fractions.

The following results were obtained on progressively fractioning (in two experiments) by the addition of the dry salt[1] to a

[1] Calculations or reference tables for the amounts of salt to be added to produce or raise a proteid solution to any desired concentration may accurately be made by employing the following formula:

$$X = \frac{v\,p\,(c_2 - c_1)}{10 - e\,p\,c_2}$$ where x is the number of gms. of salt to be added to give the required concentration, v the original volume in cc., e the increase in the volume of the solvent by 1 gm. of salt, p the gms. of salt per cc. of its saturated solution, and c_1 and c_2 are the initial and desired degrees of saturation, expressed as cc. in 10 cc. For $(NH_4)_2SO_4$ e and p may be regarded as approximately 0.54; then

$$X = \frac{v\,(c_2 - c_1)}{18.158 - 0.54\,c_2}\,;\ \text{and when } c_1 = 0,\ X = \frac{v\,c_2}{18.158 - 0.54\,c_2}$$

To raise the concentration by the addition of saturated salt solution the

liter of about 400 units antitoxic plasma. The initial dilution was 1:5. The precipitates were pressed between filters and extracted with saturated sodium chloride solution. The determinations on the filtered extracts are given per cc. of the original plasma. The results are roughly quantitative only, loss of the filtrate in pressing out the precipitated globulins being disregarded. Proteid determinations and potency tests were duplicated.

FRACTIONING OF PLASMA 305, 8/1/06.

Fractions.	Proteid per cc.	Units per cc.	Units per Gram of Proteid.
A			
0.0–3.4	0.00321	25	7788
3.4–3.6	0.00223	20	8968
3.6–3.8	0.00432	47	10879
3.8–4.0	0.00416	52	12500
4.0–4.2	0.00408	60	14705
4.2–4.4	0.00272	55	20220
4.4–4.6	0.00191	40	20942
4.6–4.8	0.00163	32	19632
4.8–5.0	0.00111	19	17117
5.0–5.6	0.00428	18	4205
B			
0.0–3.4	0.00394	27	6853
3.4–3.6	0.00219	20	9132
3.6–3.8	0.00397	45	11335
3.8–4.0	0.00336	50	14880
4.0–4.2	0.00332	60	18072
4.2–4.4	0.00255	55	21568
4.4–4.6	0.00181	40	22094
4.6–4.8	0.00147	30	20408
4.8–5.0	0.00093	18	19355
5.0–5.6	.....	18	.....

In each instance there is a progressive increase in potency as the antitoxic globulin becomes more soluble in the fractions until a concentration of the salt of about 4.2 is reached. The potency per gram remains then practically constant at about three times that of the saturated sodium chloride extract of the euglobulin fraction (0.0–3.4) until between 4.8 and 5.0 saturation; above this limit the potency per gram rapidly diminishes to a relatively very low figure. Between the 4.2 and 4.8 limits, over

amounts (cc.) of the original proteid solution and of the saturated salt solution in the mixture are calculated; also the amount of the salt solution necessary to bring the *proteid solution* to the desired concentration. Sufficient excess of saturated salt solution over that already present is added to make the required total.

half the units of the original plasma are precipitated, while the antitoxin is contained in less than one-third of the total antitoxic globulin.

The fact that the major portion of the antitoxin remained soluble at a concentration of 4.2 saturation, led us to investigate whether the protective material was mechanically precipitated with the proteid of the lower fractions. Such a result seemed *a priori* improbable because the individual fractions were at such frequent intervals and contained such a small amount of the globulin precipitate as to make hardly conceivable a mechanical inclusion of more soluble colloidal particles for more than a few minutes' duration. Our plan was to fraction the antitoxic plasma at 4.2 saturation. The lower fraction (precipitate) was to be dissolved in an added known volume of water, reprecipitated at 4.2 saturation and after standing 24–48 hours was to be filtered. The procedure was twice repeated with the precipitates obtained at 4.2 saturation. The three filtrates and the saturated sodium chloride soluble (antitoxic) globulin of the final 4.2 saturation precipitate were examined for globulin and antitoxic content. We were not able to separate by three times repeated fractioning at 4.2, the antitoxin from the lower fraction; over half the antitoxin brought down at first was pronouncedly a constituent of the precipitate, the amount in the filtrate from the final precipitation being very slight (though the potency per gram of proteid was relatively high). The protocol follows:

250 cc. of antitoxic plasma (305, 8/10/06, 300+ units per cc.) were diluted with 475 cc. of water and precipitated with 525 cc. of saturated ammonium sulphate solution. After standing 24 hours, the precipitated globulin was filtered off. To the filtrate, 1000 cc., was added 60 gms. of dry ammonium sulphate after sufficient ammonium sulphate solution had been employed to give 1500 cc. at half saturation. The resulting precipitate (4.2–5.5 saturation) was pressed out between filters, dissolved and made up to 200 cc.

The precipitate at 4.2 saturation was pressed out between filter paper, dissolved by the addition of 580 cc. of water and reprecipitated with 420 cc. of saturated ammonium sulphate solution. The total volume of the precipitated mixture was slightly over 1000 cc. After standing 24 hours, the reprecipitated globulin was filtered off from "Filtrate I" (900 cc.).

The precipitate from I was dissolved in 580 cc. of water and precipitated with 420 cc. of saturated ammonium sulphate. Filtrate II was 930 cc.

Filtrate III similarly obtained amounted to 950 cc. The globulin precipitate was extracted with 1000 cc. of saturated NaCl solution.

Determinations of proteid and antitoxic content are given per cc. of the original plasma.

	Proteid per cc.	Units per cc.	Units per Gram of Protcid.
Filtrate I.....	0.00270	40	14810
" II.....	0.00332	50	15080
" III.....	0.00075	12	16100
4.2 Ppt. gbl. (ext. NaCl)	0.01066	125	12100
4.2–5.6.	0.00685	80	11680
	0.02428	307	

Further fractioning after complete removal of the water precipitable globulin was done on 50 cc. of the globulin solution, Prep. 77 A (cf. p. 255). The fractioning was made at a dilution of the original preparation of 1:20. The results are expressed per cc. of the original undiluted preparation.

REFRACTIONATION OF PREPARATION 77A.

Fraction.	Proteid per cc.	Units per cc.	Units per Gram of Proteid.
0.0–4.0	0.0408	400	9791
4.0–4.4	0.0165	225	13667
4.4–5.0	0.0176	375	21306
5.0+	0.0018	75	41722
4.8–5.5*	0.0046	150	34783

* Made on a second 50 cc. of the same preparation.

The refractioning of 77A from which the water-acid precipitable globulin had been removed, showed a marked progressive increase in potency hand in hand with the greater solubility of the proteid.

Fraction 3.8–5.0 of Prep. 82 was refractioned without removing the water-acid precipitable globulin. The dilution was 1:10.

REFRACTIONATION OF PREPARATION 82D. (High Potency Fraction.)

Fraction.	Proteid per cc.	Units per cc.	Units per Gram of Protcid.
0.0–4.0	0.07318	600	8136
4.0–4.2	0.01779	240	13490
4.2–4.4	0.02197	260	11840
4.4–4.6	0.01232	160	12990
4.6–4.8	0.00708	90	12711
4.8–5.0	0.00511	80	15670
5.0–5.6	0.00197	90	45690
	0.13941	1510	
For 82D.	0.1341	1600	

82 D contained a globulin of rather uniform potency per gram from fractions 4.0–4.8; then a marked jump for the fraction 5.0–5.6 to about three times the original potency per gram was observed. The portion of antitoxin in the highest fractions of 77A and 82D was less than 6 per cent of the total units. Prepared for administration as is the ordinary antitoxic globulin, the resulting product would have had a potency of from 5000–6000 units per cc.

The high antitoxic potency per gram proteid of the globulin in the preparations precipitable between 4.8 and 5.6 saturation led us to attempt to obtain such a product in bulk from the antitoxic globulin preparations (Gibson) and directly from plasma. Our experiments have comprised (1) the influence of the reaction of the plasma, (2) repeated extraction (with 4.8 saturation ammonium sulphate) of the globulins to dissolve out the mechanically precipitated highly potent antitoxic substances, and finally (3) progressive denaturalization of the globulin by repeated extractions with saturated sodium chloride solution and reprecipitation with the sulphate. Our results, however, have not been encouraging. The protocols are given below:

(1) *a.* 250 cc. of antitoxic plasma (262, 10/22/06, 500 units per cc.), were diluted with 1050 cc. distilled water and precipitated at 4.8 with 1200 cc. of saturated ammonium sulphate solution. After standing three hours it was filtered and the filtrate (2250 cc.) raised to 5.0 saturation by adding 28.6 gms. of dry $(NH_4)_2SO_4$. After 24 hours at room temperature, the half saturation precipitate was filtered off, pressed between filters and made up to 225 cc. Of the 5.0 saturation filtrate, 2200 cc. were precipitated at 5.6 saturation with 96 gms. of dry $(NH_4)_2SO_4$ and filtered after 24 hours' standing at room temperature. The precipitate was pressed out and made up to 218.5 cc. in distilled water.

b. 250 cc. of the same plasma were made distinctly alkaline with $\frac{N}{10}$ NaOH and then fractioned exactly as in *a*.

c. 250 cc. of the plasma were made distinctly acid with dilute acetic acid, and then similarly fractioned.

Proteid coagulations and potency tests were made as usual.

Plasma	Fractions	Proteid per cc.	Units per cc.	Units per Grams of Proteid.
a. Native.....	4.8–5.0	0.0015	11	7333
	5.0–5.6	0.0051	20	3921
b. Alkaline....	4.8–5.0	0.0011	11	10000
	5.0–5.6	0.0047	20	4255
c. Acid.....	4.8–5.0	0.0012	11	9175
	5.0–5.6	0.0060	24	4000

(2) 500 cc. of the plasma of the bleeding employed for reprecipitation at 4.2 (305, 8/10/06, 300 units per cc.)[1] were diluted with 800 cc. of water and precipitated at 4.8 saturation with 1200 cc. of saturated ammonium sulphate solution. After standing for 24 hours, the precipitate was filtered off. Of the filtrate, 2250 cc. were precipitated, at about 5.6 saturation, with 116 gms. of dry $(NH_4)_2SO_4$; after standing, the precipitate was separated, pressed out between filter paper and made up in solution to 450 cc. with water (fraction 4.8–5.6). The moist precipitate obtained at 4.8 saturation was thoroughly suspended in about 1500 cc. of 4.8 saturation $(NH_4)_2SO_4$ and filtered after standing for two days, during which time the mixture was occasionally shaken up. The precipitate from the first filtrate (I) was reëxtracted as before, this procedure being carried on, in all, four times. The precipitate then remaining was made up with saturated NaCl solution to a volume of 1000 cc. Proteid determinations and the antitoxin tests were made on the fraction 4.8–5.6, on the four filtrates and on the NaCl extract of the residue, and are tabulated as before per cc. of the original plasma.

	Proteid per cc.	Units per cc.	Units per Gram of Proteid.
4.8–5.6 sat.....	0.00191	12½*	6316
Filtrate I.	0.00240	10*	4170
" II.	0.00160	9*	15000
" III.....	0.00026	8*	30770
" IV.	0.00030	4*	13333
Residue.....	0.01784	250	14020
	0.02431	293	

* Because of the low antitoxic and high (toxic) $(NH_4)_2SO_4$ content, the tests were made with 25 or 50 m.l.d. instead of the 100 m.l.d. ordinarily employed.

The amount of antitoxin and of proteid in the filtrates was so small that slight errors in the determinations would influence greatly the calculations of the antitoxin units per gram of proteid. Yet the results obtained on Filtrate III, when the figures on the preparations are recalled (p. 259), make it highly probable that a very small portion of the antitoxin can be separated in a much more highly potent form than is the case for the bulk of this substance.

Progressive denaturalization of the proteid as a means of separating the globulin from the antitoxic substance, if other than the serum globulin itself, has not proved successful. The method used was to extract the ammonium sulphate globulin precipitates of plasma or the antitoxic globulin preparation with saturated

[1] Cf. pp. 258 and 259.

sodium chloride solution, to filter off the insoluble globulin residue (after some days standing), and to reprecipitate the filtrate by the addition of a little over half its volume of saturated ammonium sulphate solution. The extraction of this last precipitate with the sodium chloride and the precipitation of the filtrate with ammonium sulphate followed. This procedure was carried on 4–6 times on the 4.8 residue of (2), and on two antitoxic globulin solutions, which were obtained by the sodium chloride extraction of 3.8 saturation precipitates (less potent fraction), one of which was already thoroughly denaturalized in preparation because of the accidental partial desiccation of the acidified saturated sodium chloride precipitated proteid before dialysis. The final filtrates of one of the sodium chloride extracts of the originally denaturalized antitoxic globulin preparations had a potency of almost 15,000 units per gram of proteid. The globulin solution of the 4.8 saturation extraction precipitate and the second antitoxic globulin preparation contained about 10,000 units per gram of proteid.

The injection of the antitoxic globulins of the various globulin preparations sensitizes guinea pigs to subsequent, otherwise non-fatal intraperitoneal administration of serum (Smith and Rosenau and Anderson). Injected intraperitoneally into sensitized guinea pigs, the typical convulsions produced by serum are incited, and the deaths of the animals may ensue. Rashes of the urticarial character with little or no accompanying constitutional symptoms may follow the therapeutic administration of the several fractionally precipitated antitoxic globulins. Therapeutically there is no difference in the results obtained with the equivalent unit injections of either the high (3.8 + saturation) or low (3.3 saturation) fractions of preparations 77 and 82 (pp. 255 and 256).

CONCLUSION.

From the data presented, it appears that the *saturated sodium chloride soluble* serum globulins of the higher fractions are uniformly much more potent per gram of proteid in antitoxin than are those precipitated by lower concentrations of ammonium sulphate. Between concentrations of the sulphate of 5.0 and 5.6, a small proportion of the total sodium chloride soluble glob-

ulin of the antitoxic globulin preparation (Gibson) or of a higher fraction of the same is precipitated; the solution of this globulin has a protective power of over 40,000 units per gram of proteid. The direct fractioning of the plasma, however, does not yield so potent a product; at a dilution of 1:5 of a 400 unit plasma the globulin remaining in solution at 4.2 and precipitated at 4.8 saturation has a potency of about 20,000 units per gram of proteid. It is thus practicable to prepare an antitoxic solution of over 2000 units per cc. from a relatively low plasma.

Whether or not this difference in the potency per gram of proteid is associated with the presence of non-antitoxic globulins having the same fractional precipitation limits as the protective substance remains as yet undecided. It is possible that such a variation in potency may be purely physical, associated with the size or condition of aggregation of the colloidal globulin particles—the less soluble larger masses having diminished antitoxic properties. Certainly, however, we find the antitoxin is characterized by a wide range of the precipitation limits similar to the soluble globulins, *i. e.*, in spite of repeated precipitations, a part of the antitoxin is comparatively insoluble in concentrations of ammonium sulphate in which the major portion of the protective substance readily dissolves.

In concluding the present paper, we desire to express our appreciation of Dr. Park's suggestions and helpful criticism.